Biochemical Demonstration and Immunohistochemical Localization of Calpain in Human Skin

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The biochemical properties and immunohistochemical localization of calpain, a Ca^{++}-dependent, intracellular, nonlysosomal cysteine proteinase was examined in human skin. Human epidermal calpain I was fractionated on a DEAE-cellulose column and was found to be half-maximally activated at 3.5 μM free Ca^{++} and fully activated at 10 μM Ca^{++} as measured by casein hydrolysis. Immunoelectrophoretic blotting of calpain revealed only a single band of M_r 83,000, when the blot was made with affinity-purified anti-calpain I heavy subunit IgG. Immunohistochemical staining of normal human epidermis showed that calpain I was localized in the cytoplasm of keratinocytes in the mid to upper epidermis but not in the basal cells. In untreated psoriatic epidermis, the deposition of this proteinase was visualized weakly just beneath the stratum corneum. However, remarkable staining was observed after photochemotherapy of topical psoralen plus long-wave UV irradiation. Whether the photochemotherapy induced a quantitative increase in the amount of calpain or merely made calpain more stainable by altering the membrane remains unknown. *J Invest Dermatol 86:346–349, 1986*

Increasing attention is being paid to the regulation of intracellular processes mediated by calcium ions. In addition to calmodulin and Ca^{++}-dependent phospholipid activated protein kinase c, which are recognized as important primary Ca^{++}-acceptor proteins, Ca^{++}-dependent proteinases seem to have various functions. The existence of a proteinase that is activated by both calcium ions and a sulfhydryl-reducing agent, was first found in rat brain [1]. Since then, similar Ca^{++}-dependent proteinases have been detected in different tissues as a typical intracellular nonlysosomal proteinase [2], and termed calpain (EC 3.4.22.17) [3]. Two forms of calpain, which differ in their Ca^{++} requirement, are now known to exist. Calpain I requires a low concentration of Ca^{++} for activation and calpain II requires a much higher Ca^{++} concentration. Although the biologic functions of these 2 enzymes are still obscure, a number of phenomena have been attributed to the action of calpain [4].

In the present study, we have investigated the biochemical properties and immunohistochemical localization of calpain in human epidermis using a monospecific antibody against calpain I.

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Abbreviations:

EGTA: ethyleneglycol-*bis*(β-aminoethylether)-N,N,N′,N′-tetraacetic acid

PAP: soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase antibody

PBS: phosphate-buffered saline

SDS: sodium dodecyl sulfate

MATERIALS AND METHODS

Reagents The soluble complex of horseradish peroxidase and rabbit antihorseradish peroxidase (PAP) and swine antirabbit immunoglobulin antibody were obtained from Dakopatts, Glostrup, Denmark. Swine nonimmunized serum was purchased from Dako Corporation, Santa Barbara, California. Peroxidase-conjugated goat antirabbit IgG was obtained from Cappel Laboratories, Inc., West Chester, Pennsylvania. Other chemicals of reagent grade were obtained from Wako Pure Chemical Industries, Osaka, or from Nakarai Chemicals, Kyoto, Japan.

Materials For biochemical studies, pieces of skin were obtained during plastic surgery, and stored at −70°C until use. For immunohistochemical studies, skin biopsy specimens were obtained from the back or buttock of 7 patients with untreated psoriasis vulgaris. Skin biopsy was performed on 4 of these patients after photochemotherapy, which consisted of topical application of 1% 8-methoxypsoralen (approximately 10 μl/cm^2) with subsequent long-wave UV irradiation. Patients received exposures 3 times a week with the patient's minimal phototoxic dose (1.0–3.6 J/cm^2) for 1–1.5 months before biopsy. Five pieces of normal skin were obtained during plastic surgery. They were fixed with 10% formalin, dehydrated in a graded series of ethanol, and embedded in paraffin.

Partial Purification of Human Epidermal Calpain Epidermal components were enriched with a dermatome, homogenized with 5 vol of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM ethyleneglycol-*bis*(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.25 M sucrose, in a Waring Blendor. Although there was much dermal contamination, we had found by preliminary immunohistochemical studies that little calpain was distributed in the dermis, so we used this epidermis-rich preparation for partial purification. The homogenate was ultracentrifuged at 105,000 *g* for 90 min at 4°C, and the supernatant soluton was dialyzed overnight

against 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EGTA, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 50 mM NaCl (buffer A). The concentration of protein was determined by the method of Lowry et al [5] using bovine serum albumin as the standard. The dialyzed solution (87.4 mg of protein) was applied to a column (1.5 × 5.5 cm) of DEAE-cellulose (DE 52, Whatman, Springfield, U.K.) preequilibrated with the same buffer. After extensive washing with buffer A, the adsorbed protein was eluted with a linear gradient of 50-400 mM NaCl in a total volume of 300 ml.

Assay of Calpain Calpain activity was determined with casein (Hammarsten-grade, E. Merck, Darmstadt, Germany) as a substrate. Each incubation mixture having a final volume of 1.0 ml contained 0.4% casein, 100 mM imidazole-HCl buffer, pH 7.5, 5 mM cysteine, 0.1 mM, or 5 mM $CaCl_2$. After incubation for 30 min at 30°C, the reaction was terminated by adding 1 ml of 5% trichloroacetic acid. Acid-soluble products were determined colorimetrically by the method of Ross and Schatz [6], for which 0.4 ml of the filtrate was diluted with the reagents to a total volume of 2.8 ml and the absorbance at 750 nm was read against the blank.

Immunoelectrophoretic Blotting of Calpain from Human Skin by a Monospecific Antibody Proteins were first subjected to electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (2% SDS in the initial denaturation and 0.1% in the running gels) and were then transferred to nitrocellulose paper (Schleicher & Schüll, Dassel, Germany) [7]. Nitrocellulose filters were first incubated with affinity-purified antibodies, then with a peroxidase-conjugated second antibody directed against the first antibody. Antigens were localized by the development of peroxidase staining using *o*-dianisidine as the substrate [8]. Blocking controls with nitrocellulose filters were done by preincubation of antibodies with purified porcine calpain I (5 or 10 μg/ml) at 4°C overnight.

Preparation and Purification of Antibodies Anticalpain sera were raised in rabbits using purified calpain [9,10] as reported previously [11] and DEAE-cellulose purified antibodies were then further purified by affinity chromatography as described previously [11]. The specificity of the antibodies was verified both qualitatively by a conventional double immunodiffusion method and quantitatively by enzyme-linked immunosorbent assays [12].

Immunohistochemical Staining of Skin Only the anticalpain I antibody was utilized for the immunohistochemistry. Skin sections (4 μm thick) were deparaffinized with xylene, rehydrated, incubated for 30 min with 0.3% H_2O_2 to block endogenous peroxidase activity, and incubated for 30 min with 10% nonimmunized swine serum. The sections were then incubated overnight at 4°C with affinity-purified anticalpain I heavy subunit IgG (60 μg/ml) and rinsed in phosphate-buffered saline (PBS). The sections were treated with swine antirabbit serum (diluted 20 times with PBS) and then reacted with PAP (diluted 80 times with PBS). After a 30-min incubation, calpain I was detected by the development of peroxidase staining using 3,3′-diaminobenzidine tetrahydrochloride as the substrate. The sections were counterstained by Meyer's hematoxylin and mounted. Control specimens were obtained by omitting the incubation with the first antibody. Photographs were then taken on Fujichrome 1000 film.

RESULTS

Biochemical Demonstration of Human Epidermal Calpain Fig 1 shows a DEAE-cellulose chromatograph obtained with the crude extract from human epidermis-enriched preparation. When a fixed amount of purified calpain I from porcine erythrocytes was added to each assay tube and the activity of calpain was determined in the presence of 5 mM Ca^{++}, a negative trough in activity appeared at 120 mM NaCl, indicating the elution

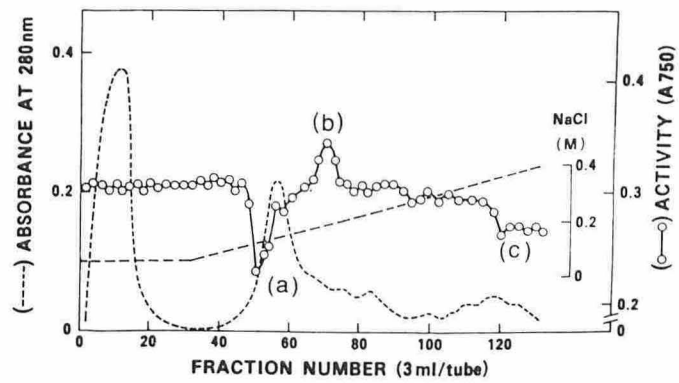

Figure 1. DEAE-cellulose chromatograph of human epidermal crude extract. Crude extract from human epidermis (87.4 mg of protein) was applied to the column (1.5 × 5.5 cm) of DEAE-cellulose (DE 52), which was developed with a linear gradient of 50-400 mM NaCl in a total volume of 300 ml. *Trough a*, calpastatin; *peak b*, calpain I; *trough c*, a possible cysteine proteinase inhibitor.

of calpastatin, a calpain-specific endogenous inhibitor protein [3] at this position. Just after the calpastatin fractions, a positive peak of activity was noted at 180 mM NaCl. When the peak fractions were pooled and examined by casein hydrolysis, they were found to be half maximally activated at 3.5 μM Ca^{++} and fully activated at 10 μM Ca^{++}, and identified as calpain I [2,3]. Calpain I activity was confirmed in the presence of Ca^{++} without exogeneously added calpain I, thus excluding the possibility that peak b in Fig 1 contains an activator of calpain I. No other calpain peaks were noted in the flow-through fractions or in the fractions that were eluted after the peak for calpain I. Fraction no. 120 seemed to represent a cysteine proteinase inhibitor other than calpastatin, since it exerted an inhibitory activity on papain. By contrast, epidermal calpastatin fractions had no effect on the hydrolysis of casein by papain, ficin, trypsin, or α-chymotrypsin (results not shown).

Immunoelectrophoretic Blotting of Calpain from Human Skin by a Monospecific Antibody The elution fractions from DEAE-cellulose column, which contained human epidermal calpain I (Fig 1*a*), were combined, and a portion corresponding to 300 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis. When the immunoblotting was made with affinity-

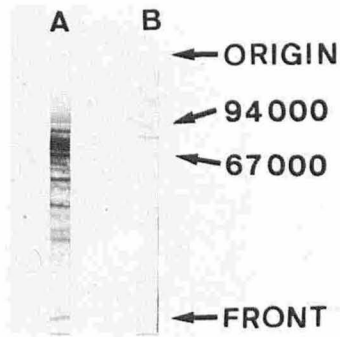

Figure 2. Immunoelectrophoretic blotting of calpain from human skin by a monospecific antibody. A portion (300 μg of protein) of the calpain fractions from DEAE-cellulose column (Fig 1) was subjected to SDS-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose filter which was stained with amido black or immunoblotted with the antibody. *Lane A*, Amido black staining of protein. *Lane B*, Immunoblot with anticalpain I heavy subunit IgG (2 μg/ml). Only a single band appeared between positions for 67K and 94K.

purified anticalpain I heavy subunit IgG, only a single band of M_r 83,000 appeared (Fig 2, *lane B*). Remarkably reduced staining was observed in blocking controls (data not shown). No cross-reactive band was found when anticalpain II heavy subunit IgG was used (data not shown).

Immunohistochemical Staining of Normal Human Epidermis by a Monospecific Antibody In normal human epidermis, cytoplasmic distribution of calpain I was less prominent in the basal portion of the epidermis and more pronounced in its granular layer. No specific staining was observed in the horny layer. Within individual epidermal cells, intracytoplasmic granular deposition was clearly visualized. No calpain I was localized in nuclear sites or cell membranes of epidermal cells (Fig 3*A*). Control specimen revealed no specific staining (Fig 3*B*). The staining was considerably reduced by preincubation of the antibody with purified porcine calpain I.

Immunohistochemical Staining of the Psoriatic Epidermis Before and After Photochemotherapy In untreated psoriatic epidermis, where the turnover rate of the epidermal cells is considered to be increased, calpain I was distributed weakly just beneath the stratum corneum as well as in scattered keratinocytes in the mid epidermis. Cytoplasmic distribution was comparable to that in normal human skin, but the deposition of calpain I was visualized weakly (Fig 3*C*). Drastic changes were noticed after photochemotherapy. Although the histopathologic findings such as elongated rete ridges and parakeratosis were unchanged, remarkable granular staining of calpain I was visualized in the mid to upper epidermis (Fig 3*D*). Basal cells were not stained even after the photochemotherapy.

DISCUSSION

In this paper, we demonstrated the presence of calpain I in normal human skin, by fractionation and characterization on DEAE-cellulose chromatography and identification by immunoelectrophoretic blotting with the monospecific antibody. We further demonstrated that calpain I was distributed in the mid to upper epidermis but not in the basal cells, that it was localized in the cytoplasm of keratinocytes, and that it was visualized in different patterns in the psoriasis before and after photochemotherapy.

Although recently the enzymologic aspects of calpain have been elucidated, the biologic functions of calpain still remain obscure. In skin specimens, we can see the physiologic as well as pathologic changes on the same section, and furthermore, several skin dis-

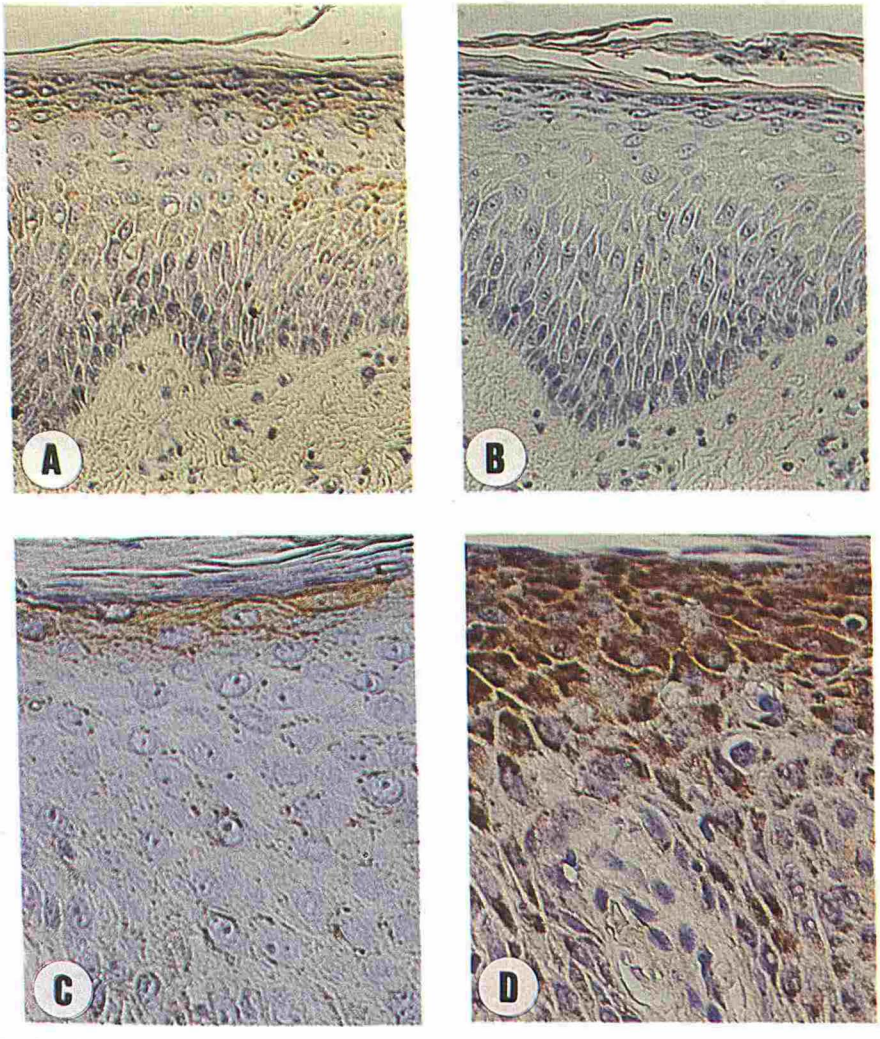

Figure 3. Immunohistochemical distribution of calpain I in human epidermis by its monospecific antibody. *A*, Normal epidermis: cytoplasmic granular deposition of calpain I was visualized in the mid to upper epidermis. No calpain I was localized in nuclear sites, cell membranes of keratinocytes, or horny layer. This staining was remarkably reduced by a preincubation of the antibody with purified porcine calpain. I. *B*, Control specimen: no specific staining was observed by omitting the incubation with the first antibody. *C*, Untreated psoriatic epidermis: calpain I was localized weakly just beneath the stratum corneum as well as in scattered keratinocytes in the mid epidermis. *D*, Psoriatic epidermis after photochemotherapy: although the histopathologic findings were unchanged, remarkable staining of calpain I was visualized in the mid to upper epidermis after the treatment, but basal cells were not stained.

eases that are thought to be natural models for pathologic conditions of proliferation and/or differentiation are readily available, thus enabling us to study, for example, the possible involvement of calpain in such processes. This is the major reason we conducted immunohistochemical distribution studies on calpain in skin biopsy samples.

The present study suggests that the differentiation rate is closely related to the calpain content in keratinocytes, though immunohistochemical staining is not a quantitative method. Skin specimens obtained from the patients with untreated psoriasis revealed that calpain I is located mainly beneath the stratum corneum, which was relatively weak when compared with normal epidermis. Of particular interest is that intense distribution of calpain I was visualized after photochemotherapy. This may mean that the distribution of epidermal calpain I can be altered by photochemotherapy, although it is not yet known from the present investigation whether this is due to the permeabilization or induction of the enzyme. However, these findings suggest that calpain is involved in the control of proliferation and/or differentiation of epidermal cells.

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